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Docket No.: 197748US0PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:

Michinobu NAKAMURA, et al.

SERIAL NO.: 09/674,280

FILED: DECEMBER 21, 2000

FOR: PROCESS FOR PRODUCING PROTEIN HYDROLYZATE

:

: EXAMINER: AFREMOVA

:

: GROUP ART UNIT: 1651

DECLARATION UNDER 37 C.F.R. §1.132

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Now comes Hideki Okamura, who deposes and states that:

1. That I am an inventor of the above-identified application.

2. That I am a graduate of Kyushu University, Japan, and received my master's degree in the field of food science, in the year 1989.

3. That I have been employed by Ajinomoto Co., Inc, for 15 years as researcher in the field of food science.
a ref
H.O. June 2, 2004

4. That I understand the English language or, at least, that the contents of the

Declaration were made clear to me prior to executing the same.

5. The distinction between liquid state and semi-solid state can be summarized as follows:

A "liquid state" is fluid, free-flowing and homogeneous state, like a solution or dispersion, with a low viscosity. In contrast, a "semi-solid state" is non-fluid, non-flowing, not uniform, and possesses a heterogeneous composition.

As described in the paragraph bridging page 4-5 of the specification, one of the objects of this invention is to "provide a process for producing hydrolyzed protein which is useful as a multi-purpose seasoning material or a multipurpose food material without contamination with germs even in the absence of a bacteriostatic substance, which process can be practiced in the industrial mass-production."

Therefore, in accordance with the present invention, raw material (e.g., wheat gluten or defatted soybean) in the culture should be completely sterilized for a fermentation process in industrial mass-production, preferably, in the absence of an added bacteriostatic substance like salt, alcohol or acetic acid.

Arising from the heterogeneous composition of "semi-solid state" or "solid state" cultures the resultant cultures are not uniform. As such, heat is not uniformly conducted in the culture. This lack of uniformity can give rise to local temperature differences that may result in microbial survival within the culture regions that have reduced temperatures. As a result, once the "sterilization" process is discontinued the surviving microbes may then repopulate the culture. Therefore, it is very difficult, or even unrealistic, to completely sterilize a "semi-solid state" or "solid state" culture on an industrial mass-production scale.

In contrast, the culture in each of each of the following are in a "liquid state:" (1) cultivating koji mold in a submerged culture fermenter-type reaction vessel; (2) mixing a dispersion of a vegetable protein material with a fungal culture of cultivated koji mold; and (3) enzymatically hydrolyzing the mixture from (2) at a first temperature with aeration and agitation and then increasing the temperature. Owing to the homogeneity of the culture, it is possible to completely sterilize (even on an industrial mass-production scale) since the culture is fluid and heat of sterilization is evenly conducted in the culture.

6. To demonstrate that the pre-hydrolysis processing provides unexpected advantages as compared to a method in which the pre-hydrolysis processing of the vegetable protein material has been omitted (i.e., U.S. 6,045,819 (Takebe et al)) the following experiments were performed (these experiments appear as Examples 1 and 4, respectively in the priority application JP 10-121029). These experiments evince that by incorporating the pre-hydrolysis processing of the vegetable protein material, the resultant protein hydrolyzate is free of contaminating microorganisms, possess a high protein activity, and possess excellent seasoning properties (e.g., taste and aroma), even in the absence of added bacteriostatic agents. At no point does the art of record (*supra*) disclose such advantages.

Example 1: Production of Amino Acid from Wheat Gluten

(Pre-processing: Emulsifying wheat gluten)

400L of city water were introduced in to a 1,000L tank connected with an emulsifier for emulsification by impact shearing, Homomicine Mill (supplied by Tokushu Kikako K.K). Water in the tank was heated. When the temperature of water reached 95°C, the operation of the emulsifier started. 20kg of a powder of active wheat gluten were charged into the tank. The wheat gluten became a completely emulsified dispersion in 30 minutes after the operation started, and at the same time, the viscoelasticity peculiar to wheat gluten dispersed. In the dispersion, neither incorporation of a coagulum (so-called dump) of wheat gluten nor inclusion of bubbles was observed at all in a slightly enlarged visual field of a microscope.

The particle diameter of the wheat gluten particles in the emulsified dispersion was on the average 150 μm (at least 10 μm ~at most 900 μm) and the concentration of the wheat gluten particles was 50 g/L.

(Pre-processing defatted soybean for liquid koji)

A defatted soybean powder obtained by roughly pulverizing unmodified defatted soybeans (supplied by Toyo Seiyu K.K.) was heated while being mixed with a mixer capable of heating procedure to conduct hot heat treatment at 98°C for 20 minutes.

(Sterilizing defatted soybean for liquid koji)

3kg of the defatted soybean powder heat-treated were charged into 200L of water having a temperature of 25°C which had been introduced into submerged culture fermenter-type reaction vessel for amino acid production while being stirred to obtain a uniform defatted soybean powder dispersion free from inclusion of bubbles. Subsequently, the dispersion was subjected to batchwise heat sterilization through heating with a superheated steam at 120°C for 20 minutes.

(Preparing liquid koji)

One percent by volume of a seed culture of a koji mold, *Aspergillus oryzae* ATCC 15240, which had been incubated after inoculating spores in a medium containing 2% sterilized defatted soybean powder, was inoculated in this heat-sterilized dispersion of defatted soybean powder. After the inoculation, the cultivation was conducted at 30°C for 24 hours with aeration at a rate of 1/4 vvm and agitation at 520 rpm to obtain liquid koji. The protease activity of the resulting liquid koji was 320 units/mL.

(Hydrolyzing wheat protein)

The total amount of the wheat gluten emulsified dispersion obtained above-mentioned method was transferred to 1 kL fermenter used in fermentation for amino acid production. Subsequently, the wheat gluten dispersion was subjected to batchwise heat sterilization through heating with a superheated steam at 120°C for 20 minutes. In the dispersion, no

inclusion of bubbles was observed at all in a slightly enlarged visual field of a microscope. When the temperature of the solution was lowered to 50°C after the completion of the heat sterilization, the half amount of the liquid koji was added thereto. The hydrolysis by enzyme reaction was conducted for 24 hours with a low speed agitation while controlling temperature of the dispersion to 45°C. In the hydrolyzed dispersion after 24 hour reaction, neither contamination with undesirable microorganisms nor growth of the microorganisms was observed.

(Post-processing hydrolyzed wheat gluten)

The obtained hydrolyzed wheat protein was relatively clear, low viscous and light yellow colored liquid with dispersing cells of koji mold. The hydrolyzate was decolored and deodorized by active carbon layer for fermentation after separation of cells of koji mold in the hydrolyzate by centrifuge. The purified liquid was concentrated at vacuum 4/r condition and spray-dried. 18 kg of the spray dried powder was obtained.

(Evaluating the spray dried wheat gluten hydrolyzate)

The spray dried wheat gluten hydrolyzate was almost odorless, light yellowish and uniformed powder and had rich and desirable taste. The result of analysis showed salt was not detected substantially in the powder. In addition, no contamination with undesirable microorganisms was substantially observed. As an overall evaluation, this spray dried wheat gluten hydrolyzate was considered to be a good flavor material for seasonings applicable to varieties of foods.

(Evaluating a spray dried wheat gluten hydrolyzate prepared as a comparison)

A fine powder of active wheat gluten prepared by the above-mentioned method was dispersed into city water having a temperature of 25°C, without dispersing into hot water.

Subsequently, the dispersion was sterilized according to the above-mentioned method. In this sterilized dispersion, incorporation of a coagulum (so-called clump) of wheat gluten was visible to naked eyes and small inclusion of bubbles was observed in a slightly enlarged visual field of a microscope. A comparison of a spray dried wheat gluten hydrolyzate by hydrolysis and spray drying. The comparison sample had strange odor and taste. A certain amount of undesirable microorganisms was observed.

(Comparing wheat gluten hydrolyzate liquid with the comparison)

The result of comparison between the wheat gluten hydrolyzate and its comparison is shown in Table 1.

Table 1: Comparison between the wheat gluten hydrolyzate and its comparison

	Process	Microorganisms	Sensory evaluation ^o	
	Hot water treatment	Contamination with microorganisms	Taste	Aroma
Wheat gluten hydrolyzate liquid	With hot water treatment	No contamination	Rich Good	Good
Comparison liquid	Without hot water treatment	Contamination* 10 ⁵ -10 ⁷ cfu/mL	Undesirable	No good

*lactobacillus, bacillus, common putrefactive microbe, etc

As shown in Table 1, the wheat gluten hydrolyzate liquid had good taste and aroma as a flavoring material. On the other hand, in the comparison liquid, contamination with other microorganisms than koji mold was apparent and it was considered to be unsuitable for a food material.

Example 4: Production of Amino Acid from Defatted Soybean

(Pre-processing defatted soybean for liquid koji)

20g of defatted soybean powder whose particle diameter is $250\ \mu\text{m} \sim 350\ \mu\text{m}$ obtained by pulverizing unmodified defatted soybeans was mixed to 1L of hot water having the temperature of 95°C in a mixer and agitated at high speed for 10 minutes. The soy protein was denatured by the hot water treatment, and air and bubbles on the surface of the defatted soybean powder were completely removed by the high speed agitation. The property of the dispersion containing the defatted soybean powder was changed to a free flowing and homogeneous dispersion with less viscous and not gelled property.

(Sterilizing defatted soybean for liquid koji)

The above-mentioned defatted soybean dispersion was put in a 5L jar-fermenter for amino acid production and subjected to heat sterilization through, heating with a superheated steam at 120°C for 20 minutes in an autoclave.

(Preparing liquid koji)

A koji mold, *Aspergillus oryzae* ATCC 15240, which has high protease producing activity was inoculated at a concentration of 10^4 spores/mL in this heat-sterilized dispersion. After the inoculation, the cultivation was conducted at 30°C for 24 hours with aeration and agitation. The protease activity of the resulting liquid koji was stable and no other microorganisms than koji mold was observed. The liquid koji was confirmed as a substantially pure culture.

(Preparing liquid koji as a comparison)

The defatted soybean dispersion with neither hot water treatment nor pulverizing treatment was heat sterilized with a superheated steam in an autoclave under the same

conditions as above-mentioned. The same koji mold spores at the same concentration were inoculated to this sterilized defatted soybean dispersion and cultivated under the same conditions. The protease activity of the resulting liquid koji as a comparison did not reach the initial level and lowered during the cultivation. Further, contamination with microorganisms other than koji mold was terrible.

The comparison between the liquid koji of this invention and the comparison liquid koji is shown in Table 2.

Table 2: The comparison between the liquid koji and its comparison

	No.	Pre-processing		Property of Liquid koji	
		Pulverization Particle diameter	Hot water treatment	Other Microorganism than koji mold	Protease Activity (U/mL)
Liquid koji of Invention	1	With Treatment 50-150 μm	With Treatment 95°C for 10 minutes	Not exist Not detectable	250-260
	2	With Treatment 50-150 μm	Without Treatment Ambient temperature	Exists 10 ³ cfu/mL	100-240
Liquid koji of Comparison	3	Without Treatment Flake	With Treatment 95°C for 10 minutes	Exists 10 ² cfu/mL	100-200
	4	Without Treatment Flake	Without Treatment Ambient temperature	Exists 10 ³ cfu/mL	50-100

As shown in Table 2, in the liquid koji comprising koji mold and the defatted soybean dispersion with pulverization, hot water treatment and heat sterilization in an autoclave, neither contamination with undesirable microorganisms nor growth of the microorganisms was observed. The protease activity was more than 5 times of that of the comparison.

7. I declare further that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

8. Further Declarant saith not.

Hideki Okamura

岡村 英喜

Date

June. 2. 2004